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Research article

Down-regulation of IRF3 expression in Relapse-Remitting MS patients

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Abstract: *Background:* Relapsing-Remitting (RRMS) is the most common Multiple Sclerosis disease course. Interferon regulatory factor 3 (IRF3) as major regulators of immune system genes plays a critical role in the activation of type I interferons promoters, in particular IFNβ promoter. Hence we aimed to evaluate the expression rate of IRF3 in RRMS patients under different type of IFNβ treatment. *Material and methods:* In the present study total of 100 subjects participated. Blood samples of 25 patients with RRMS newly diagnosed who have not been treated with interferon components, 25 patients with RRMS treated with Interferon beta-1α (B1a), 25 patients with RRMS treated with Interferon beta-1β (B1b) and 25 control samples were collected. The samples were transferred at standard conditions to the Cellular and Molecular Research Center of Shahrekord University of Medical Sciences, RNA was extracted and converted to cDNA. To evaluate the expression of IRF3 the Real-Time PCR method using SYBR Green dye was done. The level of gene expression was measured by a comparative threshold cycle formula. The obtained data were analyzed using SPSS v15 software. *Results:* In the study we compared the IRF3 mRNA expression of all subjects in association with gender, which no significant difference was seen ($P > 0.05$). Also assessment of the gene mRNA level in study groups revealed that the B1b, B1a and new case group had the lowest expression respectively. Moreover, comparison of the mRNA level between new case and B1b groups showed remarkable difference ($P < 0.05$). According to age and sex factors, no

remarkable differences between study groups were seen (*P > 0.05*). *Conclusion:* Perhaps the IFNβ recombinants decreases the IRF3 expression as a negative feedback mechanism. Overall the data reported here, supports the previous studies in important role of IRF3 in autoimmune inflammatory disease of CNS and Multiple Sclerosis.

Keywords: multiple sclerosis; IRF3; IFNβ

1. Introduction

Multiple sclerosis (MS) is a chronic immune-mediated disorder of central nervous system resulting from both genetic and environmental factors. The MS characterized by inflammation and neurodegeneration with significant social and economic impact. It usually affects young individuals between 20 to 40 years old which about 60% of cases are females [1]. The worldwide prevalence of MS varies in different geographical regions and has increased in last decades. Global estimation revealed that the disease affects more than 2.5 million people. In Iran the MS has a growing prevalence rate (51.52 per 100,000) [2].

The dominant hypothesis in MS pathophysiology considers an important role for auto-reactive T cells in which these cells leading series of events such as increment of pro-inflammatory cytokines, adhesion molecules, metalloproteinases, blood-brain barrier damage and as a result infiltration of immune cells to CNS [3,4]. According to international panel of neurologists the MS has four distinct clinical patterns, which the most common subtype is the Relapsing-Remitting MS (RRMS) that accounts for approximate 85% of cases. In this form, an acute phase followed up by partial or full recovery [5].

IRF3 as a member of interferon regulatory factor family is one of the major regulators of immune system genes with well-known role in antiviral immunity [6]. Pathogens or synthetic ligands stimulate pattern recognition receptors that resulting in phosphorylation of IRF-3. Afterward the factor translocates to nucleus in order to induction of Type I interferons transcription and results in the activation of type I interferons promoters, in particular IFNβ promoter. Secreted IFNβ through JAK-STAT signaling pathway induces IRF7 that as a positive feedback loop amplifies multiple subtype of type I IFN [6–8].

Of type I interferons, beta interferons are the first line of disease-modification treatments (DMTs) that have proved efficacy for treatment of RRMS patients. Recombinant IFNβ has 3 different pharmaceutical formula: IFNβ-1a, 6 MIU (30 μg) (Avonex), IFNβ-1a (22 and 44 μg) (Rebif), and IFNβ-1b, 8 MIU (250 μg) (Betaferon). Although the precise mechanism of action of these immunomodulatory drugs in MS has not been clarified, but it seems that generally they shift cytokine profile from pro-inflammatory to anti-inflammatory [9]. A baseline level of IFN for maintain type I IFN signaling is needed. High percentage of MS patients have low serum IFNs, so this may be the reason of low responses to IFNβ therapy [10].

Limited studies investigated the role of IRFs in multiple sclerosis. According to earlier studies IRF-3 modulates neuroinflammatory responses [11]. Tarassishin et al. investigated the critical role of IFR-3 in microglia phenotype change in CNS, the results showed that IRF3-overexpressing microglia upregulate key anti-inflammatory cytokines and downregulate proinflammatory cytokines [6].

Touil et al. reported that suppression of RRMS in murine model of MS by polyinosinc-polycytidylic acid (P I:C) is associated with the induction of IFNβ. Their data indicated that P I:C exerts its anti-inflammatory effects through TLR3 stimulation followed by translocation of IRF3 that resulted in IFNβ production and increased level of TNF-α [12].

Considering previous studies about the central role of IRF3 in signal transduction cascades of type I IFN and immune-mediated disease, we have decided to evaluate the expression rate of IRF3 in RRMS patients under different type of IFNβ treatment.

2. Material and methods

2.1. Subjects

This case-control study was conducted on the patients referring MS Center and Kashani Hospital, Shahrekord, Iran from 2017 to 2018. A total of 100 individuals (21 males, 79 females) participated in the research including 25 new cases of RRMS patient, 25 RRMS patients treated with IFNβ-1a (B1a), 25 RRMS patients treated with IFNβ-1b (B1b) and 25 control subjects. The inclusion criteria for study group were as follows: meeting the 2017 revisions of the "McDonald" Criteria [13] for definite diagnosing of MS, a disease course featuring relapses and remissions. Also the exclusion criteria for this group were as follows: history of any autoimmune disease, chronic infection, and use immunomodulatory therapy or immunosuppressive drugs. Study was approved by the ethics committee of Shahrekord University of Medical Sciences and all patients provided written informed consent.

2.2. Total RNA extraction and cDNA synthetize

All participants' blood samples (3ml) were collected in EDTA tube. Total RNA from peripheral blood cells was extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. Briefly the blood was incubated with erythrocyte lysing buffer. Then cloudy suspension was washed and centrifuged at 400 g for 10 minutes. The cellular pellet of leukocytes was lysed with RTL solution. The cell emulsion was loaded on spin columns and centrifuged. Later 70% ethanol added to the homogenized lysate and pipetted into new spin columns and centrifuged. On the next step the solution was washed with DW. Then the RPE buffer added to column and centrifuged at high speed (14,000 RPM). At the end the spin column transferred to microcentrifuge tube and 30–50 μL of RNase-free water were added directly to the membrane and centrifuged. Extracted RNA were treated with DNase and reverse-transcribed to cDNA, according to the Transcription kit instructions (QIAGEN, Germany).

2.3. Real-time quantitative PCR

To evaluate the expression level of IRF3 in study groups, quantitative real-time PCR analyses were performed using specific designed primers and SYBER Green PCR Master Mix (QIAGEN, Hilden, Germany) in Corbett 3000 (Corbett, Sydney, Australia) detection system. The sense and anti-sense primers for IRF3 were as follows: sense GGACGCTCACCACGCTAT anti-sense: GCACAACCTTGACCATCACGA. 1 μL of synthesized cDNA products were added into PCR

reaction mix containing 5 µL SYBR Green master mix, 0.5 µL of each primer and 3µl of nuclease-free water. The PCR process was programmed as 94 °C for 45 seconds (Taq polymerase activation), 94 °C for 15 seconds (denaturation), 60 °C for 20 seconds (annealing), and 72 °C for 25 seconds (extension). Samples were run in triplicate and *GAPDH* gene used as an endogenous control. Expression levels of target genes were calculated using a comparative threshold cycle formula. That is, the expression level of target genes to reference genes in treated samples compared to the normal controls was calculated through $2^{-\Delta\Delta CT}$ formula:

$$\Delta\Delta CT = \Delta(CT_{\text{target gene}} - CT_{\textit{control group}}) - \Delta(CT_{\text{target gene}} - CT_{\textit{GAPDH}}) \quad (1)$$

2.4. Statistical analysis

Data of mRNA expression were analyzed by SPSS v15 software. Relative expression of IRF3 gene between patient groups calculated using the Student's t-test and Chi-square test. Data were reported as mean ±SD. A *p value* of less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic data of participants

In this research age and sex ratio distribution showed that from 100 subjects 21% were male (mean age: 32.05 ± 9.9 years) and 79% were female (mean age: 30.3 ± 7.3 years). In the present study 25 age- and sex-matched healthy control subjects and 75 patients were recruited. Our 75 RRMS patients diagnosed according to the revised McDonald criteria[13], consist of 18 males and 57 females (Table 1). Comparison of subjects mean age according to sex ratio, did not show any significant difference ($P > 0.05$). Also evaluation of patients groups mean age showed no significant differences compare to control group ($P > 0.05$). The age and sex distribution in our patients and control groups were normalized.

Table1. Demographical and clinical characteristics of participants.

Groups	Number	Age (mean ± SD)	Female / Male
Control	25	30.4 ± 7.5	22 / 3
New case	25	27.3 ± 7.5	18 / 7
B1a	25	33.9 ± 8.4	18 / 7
B1b	25	31.6 ± 7.2	21 / 4

3.2. Lower IRF3 gene expression in RRMS patient groups

The mRNA expression of IRF3 was evaluated by real-time PCR method in study groups. The mean of IRF3 gene expression in all subjects (100) was 3.75 ± 3.19. Also comparison of the mRNA expression of all subjects in association with gender, showed no significant difference ($P > 0.05$). The IRF3 mRNA level was assessed in study groups. The results revealed that in comparison to control group, the B1a, B1b and new case groups had lower mRNA expression respectively. IRF3 mRNA level in B1b and B1a groups compared to control group was remarkably lower. Moreover,

the gene expression in new case group showed significant difference with B1b group. In comparison of IRF3 expression level in B1a group to new case and B1b groups, no remarkable difference was seen (Figure 1). In this research according to age and sex factors, no remarkable differences between study groups were seen.

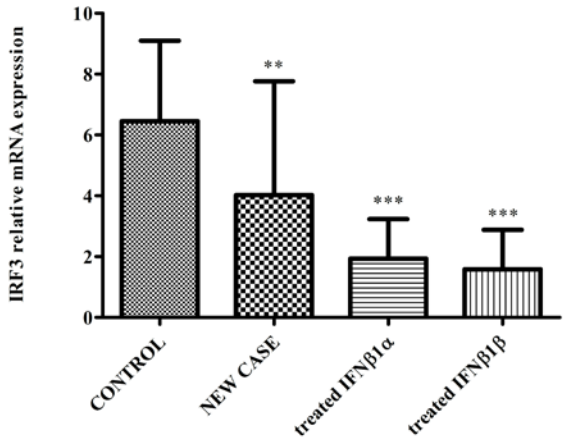

Figure 1. The relative expression of IRF3 in different study groups evaluated by by Real-time RT-qPCR method. Samples were run in triplicate and *GAPDH* gene used as an endogenous control. The mRNA expression of target gene in all study groups were calculated using the $2^{-\Delta\Delta CT}$ formula. $\Delta\Delta CT = \Delta(CT_{\text{target gene}} - CT_{\textit{control group}}) - \Delta(CT_{\text{target gene}} - CT_{\textit{GAPDH}})$. The mean expression levels of IRF3 was assessed in study groups. The results revealed that the control group had the most mRNA expression and the new case, B1a and B1b groups had lower expression respectively. *** Indicates a significance level below 0. **Indicates a significance level below 0.05.

4. Discussion

The exact cause of MS disease has not been clearly clarified yet, however genetic recognized as the major contributor factor. Beside genetics, nucleic and mitochondrial DNA defects, Viral infections, hypoxia, oxidative stress, lack of light or low levels of vitamin D and increased macrophages (microglia) and lymphocytes in the brain may cause the development of MS [14]. In the present study we evaluated the IRF3 gene expression in RRMS patients compared with controls.

In the present study, 21% of all subjects were male and 79% were female, and the average age of the patients did not show a significant difference in association with their sex. The mean age evaluation revealed that B1b and new case group, respectively, had the highest and lowest mean age. There was a significant difference between the two groups regarding age factor. Pajouh et al. identified the frequency and characteristics of dysphagia in multiple sclerosis patients of Khuzestan M.S society. Their study consisted of 21 male and 84 female with mean age of 33.89 ± 8.56 years [15]. The results of our study in relation to the sex ratio and mean age of the patients was in consistent with Pajouh et al. study. Also in a research by Poorjavad et al. the association factors with swallowing disorders in patients with multiple sclerosis was assessed. In their research 19.8% of MS patients were male and 80.2% were female, and the mean age of subjects were 34 ± 9.3 years [16].

Our study results in relation to age and sex ratio was in line with their study. Also present study indicates higher prevalence of MS in females than in males with more frequent develop of RRMS in females. It seems that differences in immune and nervous system between women and men as a consequence of gonadal hormones effects, also genetic and life style affects the MS incidence among women.

The results of present study showed that IRF3 mRNA level in B1b, B1a and new case groups were respectively lower compared to control group Schultz et al. evaluated the role of IRF3 and IRF7 deficiencies in the mice with Sindbis virus (alpha virus that cause encephalomyelitis). The results revealed that IRF3-/- mice compared to wild type mice developed persistent neurological deficits, more inflammation in spinal cord and higher levels of IFNγ mRNAs [17]. Another study by Tarassishin et al. explored the consequence of adenovirus-mediated IRF3 gene transfer (Ad-IRF3) in primary human astrocytes. They showed that IRF3 transgene expression suppresses proinflammatory cytokine gene expression upon challenge with IL-1/IFNγ and alters astrocyte activation phenotype from a proinflammatory to an anti-inflammatory one, akin to an M1 to M2 switch in macrophages. Also Ad-IRF3 suppressed the expression of microRNA-155 and its star-form partner miR-155*and immunoregulatory miRNAs which highly expressed in multiple sclerosis lesions [18]. As we mentioned different IFNβ compounds are used as immunoregulators in MS patients. According to Feng et al. study cultured mononuclear immune cells of MS patients with active clinical progression are resistant to IFNβ and operation of responses needed a high doses of IFN in MS patients' active clinical progression [19]. Also in vivo study by Panitch et al. revealed that IFNβ therapy in early RRMS patients than those with later stage of MS is more effective [20]. Our results along with this studies showed difference in IRF3 gene expression in IFNβ treated groups. According to our finding time passing and progression of disease could alter the IRF3 gene expression. Also lower levels of IRF3 mRNA expression in IFNβ treated groups compared to new case group were seen. These findings may show that the interferon beta component exert their effects through alternation of IRF3 signaling pathways [20]. It appears that this drugs decrease the production and activity of IL-1 cytokine and therefore reduce inflammatory responses in CNS. The exact mechanism of action of IFNβ recombinants in altering IL-1 have not been fully elucidated but some studies shown that this drugs through their effect on Toll-Like Receptors (TLRs) and signaling molecules led to a decrease in IL-1 production [21] and also effects on NLRs results in decreased activity of this cytokine [22]. In a study by Downer et al the effects of a synthetic cannabinoid on TLR signaling, with particular focus on the molecular mechanism controlling the induction of IFNβ were assessed. They introduce the synthetic cannabinoid, *R*(+)WIN55,212-2 as a novel regulator of TLR3 and TLR4 signaling by mechanism of inhibiting the pro-inflammatory signaling axis that triggered by TLR3 and TLR4, whereas selectively augmenting TLR3-induced activation of IFN regulatory factor 3 (IRF3) and expression of IFNβ. Furthermore that cells from MS patients were especially sensitive to *R*(+)WIN55,212-2 in terms of increased expression of endogenous IFNβ and this strongly indicated this mechanism has relevance to treatment of MS [23]. In the present study the IRF3 expression in new case group compared to control group was significantly lower. It seems that immune system responses in the early stage of the disease in a negative feedback suppresses the IRF3 expression in order to regulate the inflammatory conditions. This finding is in consistence with previous studies in effectiveness of early MS diagnosis and treatment in improvement of mortality [24]. Because the immune system at an early stage tries to trigger inhibitory mechanisms for modulating inflammatory factors.

Recent study reported a crucial role of IRF3 in development of TH17 responses and induction of EAE. So according to our findings probably recombinant IFNβ products exert their effects through lowering the IRF3 factor that followed by decreased inflammatory responses. Overall our data supports the important role of IRF3 in immunoregulation and immune-mediated disease and further study is needed to evaluate the alternation of gene expression of other proteins in IRF3 signaling.

5. Conclusion

Determination of IRF3 mRNA level in blood of RRMS patients showed a reduction of the gene expression in new case, B1a treated and B1b groups respectively compared to control group. Perhaps the IFNβ recombinants decreases the IRF3 expression as a negative feedback mechanism. Overall the data reported here, supports the previous studies in important role of IRF3 in autoimmune inflammatory disease of CNS and Multiple Sclerosis.

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Conflict of interest

The author declares no conflicts of interest in this paper.

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